

Manuscript Number:

Title: Unexpected allergenic 11S globulin (Act d 12) and 2S albumin (Act d 13) from kiwi seeds as novel masked relevant components in kiwifruit allergy

Article Type: Original Article

Section/Category: Food, Drug, and Insect Sting Allergy and Anaphylaxis

Keywords: food allergy, kiwifruit allergy, kiwi seeds, allergen, 11S globulin, 2S albumin, Act d 12, Act d 13, component-resolved diagnosis, cross-reactivity

Corresponding Author: Mr. Oscar Palomares, PhD

Corresponding Author's Institution: Universidad Complutense

First Author: Sofia Sirvent, PhD

Order of Authors: Sofia Sirvent, PhD; Belén Cantó, BSc; Javier Cuesta-Herranz, MD, PhD; Francisca Gómez, MD; Natalia Blanca, MD, PhD; Gabriela Canto, MD, PhD; Miguel Blanca, MD, PhD; Rosalía Rodríguez, PhD; Mayte Villalba, PhD; Oscar Palomares, PhD

Manuscript Region of Origin: SPAIN

Abstract: Background: Kiwifruit is a common cause of IgE-mediated food allergy. Component-resolved diagnosis of kiwifruit allergy suggests that relevant allergenic components are still undiscovered. The presence of allergens in kiwi seeds has not been investigated in detail so far.

Objective: To increase the diagnostic sensitivity of kiwifruit allergy by identifying new allergens that might have been masked due to their specific location in seeds.

Methods: Fifty-five kiwifruit allergic patients were included in the study. Immunoblotting, proteomic tools and Edman degradation were used to identify and purify IgE-reactive proteins from kiwi seeds. Circular dichroism, mass spectrometry, immunoblotting, enzyme-linked immunosorbent assay, skin prick test and IgE-inhibition experiments were used for structural and immunological characterization of purified allergens.

Results: Two novel allergens, an 11S globulin (Act d 12) and a 2S albumin (Act d 13) have been identified and purified from kiwi seeds. Both purified molecules display the typical structural features reported for other members of their corresponding protein families. Act d 12 and Act d 13 retain IgE-binding capacity and are recognized by 70.9 % and 18.2 %, respectively, of the tested kiwifruit allergic patients. Both purified allergens show in vivo allergenicity and display in vitro IgE-cross reactivity with homologous counterparts from peanut and tree nuts.

Conclusion: The identification of 11S globulin Act d 12 and 2S albumin Act d 13 as novel relevant allergens from kiwi seeds and their availability as well-defined molecules might contribute to improve the diagnosis of kiwifruit allergy.

Sirvent et al.

TITLE PAGE

Original article

Unexpected allergenic 11S globulin (Act d 12) and 2S albumin (Act d 13) from kiwi seeds as novel masked relevant components in kiwifruit allergy

Sofía Sirvent, PhD,^a Belén Cantó, BSc,^a Javier Cuesta-Herranz, MD, PhD^b, Francisca Gómez, MD,^c Natalia Blanca, MD, PhD,^d Gabriela Canto, MD, PhD^d Miguel Blanca, MD, PhD^c Rosalía Rodríguez, PhD,^a Mayte Villalba, PhD,^a and Oscar Palomares, PhD^a

^aDepartment of Biochemistry and Molecular Biology, School of Chemistry, Complutense University of Madrid, Spain

^b Allergy Service, Fundación Jiménez Díaz, Madrid, Spain

^c Allergy Service, Hospital Carlos Haya, Málaga, Spain

^d Allergy Service, Hospital Infanta Leonor, Madrid, Spain

Corresponding Author: Oscar Palomares, PhD
Department of Biochemistry and Molecular Biology,
Chemistry School, Complutense University of Madrid,
Ciudad Universitaria s/n, 28040 Madrid, Spain.
Telephone: + 34 913944161
Fax: + 34 913944159
Email: oscar.palomares@quim.ucm.es

This work was supported by grants: The authors' laboratories are supported by the Spanish Ministerio de Economía y Competitividad (MINECO) SAF2011-26716 and SAF2008-04053 and Fondo de Investigación Sanitaria (FIS) RD12/0013/0015. OP is a Ramon y Cajal Scholar funded by MINECO and the European Social Fund.

ABSTRACT

Background: Kiwifruit is a common cause of IgE-mediated food allergy. Component-resolved diagnosis of kiwifruit allergy suggests that relevant allergenic components are still undiscovered. The presence of allergens in kiwi seeds has not been investigated in detail so far.

Objective: To increase the diagnostic sensitivity of kiwifruit allergy by identifying new allergens that might have been masked due to their specific location in seeds.

Methods: Fifty-five kiwifruit allergic patients were included in the study. Immunoblotting, proteomic tools and Edman degradation were used to identify and purify IgE-reactive proteins from kiwi seeds. Circular dichroism, mass spectrometry, immunoblotting, enzyme-linked immunosorbent assay, skin prick test and IgE-inhibition experiments were used for structural and immunological characterization of purified allergens.

Results: Two novel allergens, an 11S globulin (Act d 12) and a 2S albumin (Act d 13) have been identified and purified from kiwi seeds. Both purified molecules display the typical structural features reported for other members of their corresponding protein families. Act d 12 and Act d 13 retain IgE-binding capacity and are recognized by 70.9 % and 18.2 %, respectively, of the tested kiwifruit allergic patients. Both purified allergens show *in vivo* allergenicity and display *in vitro* IgE-cross reactivity with homologous counterparts from peanut and tree nuts.

Conclusion: The identification of 11S globulin Act d 12 and 2S albumin Act d 13 as novel relevant allergens from kiwi seeds and their availability as well-defined molecules might contribute to improve the diagnosis of kiwifruit allergy.

CLINICAL IMPLICATIONS: Kiwi seeds are a source of relevant allergens for kiwifruit allergic patients. The inclusion of Act d 12 and Act d 13 in component-resolved diagnosis of kiwifruit allergy could reduce the number of misdiagnosed patients.

CAPSULE SUMMARY: We have purified and characterized two novel relevant allergens from kiwi seeds (Act d 12 and Act d 13) previously overlooked in the context of kiwifruit allergy that might well contribute to improve the diagnostic sensitivity.

KEY WORDS: food allergy, kiwifruit allergy, kiwi seeds, allergen, 11S globulin, 2S albumin, Act d 12, Act d 13, component-resolved diagnosis, cross-reactivity

ABBREVIATIONS:

CRD: Component-resolved diagnosis

OAS: Oral allergy syndrome

SPT: Skin prick test

ELISA: Enzyme-linked immunosorbent assay

OD: Optical density

MS: Mass spectrometry

Introduction

Kiwifruit (*Actinidia deliciosa*) is a common cause of IgE-mediated food allergy.¹⁻³ The prevalence of kiwifruit allergy has significantly increased over the last years, being currently considered among the top 10 food allergies.^{4, 5} The main clinical manifestations triggered by kiwifruit ingestion range from mild local oral allergy syndrome (OAS), which is frequently associated to birch and grass pollen allergy,⁶ to severe systemic reactions including anaphylaxis which is mainly experienced by patients monosensitized to kiwifruit.⁷ ⁸ Kiwifruit allergy has been also associated to latex allergy^{9, 10} and to occupational baker's asthma.¹¹

The gold standard approach to accurately diagnose kiwifruit allergy remains double-blind placebo-controlled food challenges, but the main inconveniences to this approach include the difficulty to properly mask kiwifruit and the risk of anaphylactic reactions.^{12, 13} Different attempts at improving the *in vivo* and *in vitro* diagnosis of kiwifruit allergy have been performed.^{1-3, 5} Several studies demonstrated prick to prick test with fresh kiwifruit as a highly sensitive method for diagnosis of kiwifruit allergy (83 % to 100 %) with low specificity (around 31 %). In contrast, *in vivo* SPT and *in vitro* serum specific-IgE determinations with commercially available kiwifruit extracts improved specificity (40%-45 %) but showed lower sensitivity (17 %-60 %) than prick to prick tests. Recent studies demonstrated component-resolved diagnosis (CRD) with purified natural and recombinant kiwifruit allergens as an alternative not only for increasing the diagnostic sensitivity but also to link specific IgE-sensitization patterns with clinical features.^{1, 2, 14}

Up to date, 11 kiwifruit allergens from distinct protein families and with different clinical relevance have been described according to the International Union of Immunological Societies (IUIS) allergen nomenclature subcommittee (www.allergen.org).¹

⁵ These allergens include Act d 1 (Actinidin, a cysteine protease), Act d 2 (thaumatin-like protein), Act d 3 (40 kDa glycoallergen), Act d 4 (cystatin), Act d 5 (kiwellin), Act d 6 (pectin

methylesterase inhibitor), Act d 7 (pectin methylesterase), Act d 8 (Bet v 1 homologue protein), Act d 9 (profilin), Act d 10 (non-specific lipid transfer protein (nsLPT)) and Act d 11 (major latex protein/ripening-related protein). The use of purified kiwifruit allergens in CDR approaches improved the diagnostic sensitivity up to 65% with respect to commercial kiwifruit extracts,¹ but there are still a large number of kiwifruit allergic patients that did not show reactivity to any of the allergens included in that work, suggesting that other highly relevant allergenic proteins from kiwi might remain still undiscovered. At this regard, almost all the kiwifruit allergens described so far have been identified in the kiwi pulp and only Act d 10 (LTP) has been reported to be also contained in kiwi seeds.¹⁵ There are several key aspects that make plausible the existence of additional relevant allergens in kiwi seeds that might have been overcome in the context of kiwifruit allergy: i) kiwi seeds are not usually removed from the kiwifruit, and therefore, they are also ingested together with the kiwi pulp; ii) different seeds from other species are well-recognized potent inducers of food allergy;¹⁶⁻¹⁹ iii) seeds contain high levels of very stable allergenic proteins such as 2S albumins, 7S vicilins or 11S globulins able to sensitize at the gastrointestinal level and involved in cross-reactivity.²⁰⁻²² The aim of this study was to identify and characterize new relevant allergens specifically present in kiwi seeds that might help to improve the current diagnosis of kiwifruit allergy.

METHODS

Patients allergic to kiwifruit

Kiwifruit allergic patients were diagnosed following the criteria previously reported²³ and recruited from the Allergy Service of 3 different centers in Spain: Hospital Carlos Haya from Málaga and Hospitals Fundación Jiménez Díaz and Infanta Leonor from Madrid. The study was approved by the Ethic Committee of the 3 Hospitals, and written informed consent was obtained from all subjects. During the patient consultation a questionnaire gathering relevant clinical information was filled out by an allergist and all the patients were tested by skin prick test (SPT) for a battery of foods (including kiwifruit) and aeroallergens commonly sensitizing in the Mediterranean area with commercial available extracts (ALK-Abelló and LETI). Serum samples from all the patients were collected for specific IgE determinations and storage at -20°C. Sera from non-atopic and pollen-allergic patients without food allergy were used as controls.

Fully detailed Methods on the preparation of home-made kiwi extracts (total, pulp and seeds), skin prick test, purification of Act d 12 and Act d 13, immunoblotting, ELISA, analytical, circular dichroism and proteomic procedures are described in the materials and methods section of the **Online Repository**.

RESULTS

Clinical features of patients allergic to kiwifruit

The general clinical characteristics of the 55 kiwifruit allergic patients included in this study are summarized in Table I. Median age of the patients was 34.8 yrs [25.0-42.0], with a predominance of females (39 vs 16). Thirty-four patients (61.8 %) referred exclusively local symptoms and 21 patients (38.2 %) developed immediate systemic reactions after the ingestion of kiwifruit. The most frequent reported symptom was OAS (61.8 %) and there were also 9 patients suffering from anaphylaxis (16.3 %). Other reported symptoms included angioedema (14.5 %), generalized urticaria (5.4 %), contact urticaria (3.6 %) and gastrointestinal disorders (1.8 %). Twelve patients (21.8 %) were only allergic to kiwifruit, tolerating the remaining plant-derived foods at the moment of the study. The other 43 patients (78.2 %) suffered symptoms with other plant foods, being members of the *Rosaceae* family the most frequently reported allergies (28 patients) followed by the tree nuts (23 patients) and peanut (17 patients). Thirty-two patients (58.2 %) were also allergic to pollens. SPT to all the above mentioned food allergenic sources and the most common sensitizing food and pollens in the Mediterranean area²⁴ were performed (data not shown). Only 28/55 patients included in this study (50.9 %) showed positive SPT with commercial kiwifruit extract. We grouped the kiwifruit allergic patients according to positive (group 1) or negative (group 2) SPT to kiwifruit extract (Table I). Only 14/28 patients from group 1 (50.0 %) and 5/27 from group 2 (18.5 %) had IgE to kiwifruit extract by ELISA.

Detection of IgE-reactive proteins in kiwi seeds

We assayed two pools of sera from patients included in the group 1 and 2, respectively, against home-made total kiwifruit (pulp and seeds), pulp kiwifruit and kiwi seeds extracts in immunoblotting for detection of IgE-reactive proteins. The protein content of total and pulp kiwifruit extracts was very similar (lane CBS, Figure 1). Two main proteins bands of around

51 and 12 kDa were observed in kiwi seeds but not in total and pulp kiwifruit extracts (lane CBS, Figure 1). Both proteins were recognized by the two pools of sera (Figure 1), suggesting that these IgE-reactive proteins might represent two novel allergens from kiwi seeds. The pool of sera from group 2 (negative SPT to kiwifruit extract) exclusively reacted against the allergens contained in kiwi seeds extract, whereas the pool of sera from group 1 (positive SPT to kiwifruit extract) also reacted to other proteins bands of around 20-22, 27-30 and 36 kDa contained in total and pulp kiwifruit but not in kiwi seeds extract.

We tested the sera from all the patients against kiwi seeds extract (Figure 2) and total kiwifruit extract (Figure E1). Thirty-nine out of the 55 patients (70.1 %) reacted to the protein of 51 kDa in kiwi seeds (Figure 2), 21/28 (75 %) and 18/27 (66.7 %) from groups 1 and 2, respectively. Ten out of the 55 patients (18.2 %) showed positive response to the protein of 12 kDa in kiwi seeds, 4/28 (14.3 %) and 6/27 (22.2 %) from groups 1 and 2, respectively. Seven patients also reacted against an IgE-reactive protein of around 23 kDa in kiwi seeds. Twenty out of the 28 patients from group 1 (71.4 %) showed specific IgE to at least one allergen contained in total kiwifruit extract, whereas only 5/27 (18.5 %) from group 2 were positive (Figure E1). The same result was obtained for kiwifruit pulp extract (data not shown). The IgE-reactive proteins of 51 and 12 kDa in kiwi seeds extract were not contained in total nor pulp kiwifruit extract.

Identification and structural characterization of two novel allergens in kiwi seeds:

11S globulin (Act d 12) and 2S albumin (Act d 13)

The IgE-reactive protein bands of 51 and 12 kDa contained in kiwi seeds were separated by SDS-PAGE (Figure 3A, inset), excised from the gel and digested with trypsin. The obtained peptides were analyzed by MALDI-TOF-MS and some of them (shaded arrows in MS-profile) subjected to fragmentation and MS/MS analysis. The identified peptides from each protein are indicated in the MS-profile (Figure 3A). The comparison of these peptides

with those from databases (Figure E2) allowed the identification of the proteins of 51 and 12 kDa as an 11S globulin and a 2S albumin, respectively, which were named Act d 12 (UniProt accession number C0HJF9) and Act d 13 (UniProt accession number C0HJG0) according to the IUIS allergen nomenclature subcommittee.

Both allergens from kiwi seeds were purified to homogeneity (Figure 3B) following the sequential chromatographic steps described in the result section of the **Online Repository** (Figure E3). Edman degradation of the N-terminal of purified Act d 12 rendered the double sequence, NRQPSKI and GLEETI. These sequences were 100 % and 83.3 % identical to those encompassed from residues 1 to 7 and 256 to 261, respectively, in the 11S globulin from *Actinidia chinensis* (UniProt accession A0EM47) (Figure E2A), which correspond to the N-terminal sequences of the large (32 kDa) and the small (20 kDa) subunits, respectively, of the allergen that are separated under reducing conditions (Figure 3B). The 2S albumin Act d 13 also consisted of two polypeptide chains of around 8 and 4 kDa disrupted by reducing agents (Figure 3B), but in this case we only obtained a single sequence, GPQQQHRL, by Edman degradation. This sequence showed 63% identity with the N-terminal (positions 41 to 49) of the large chain of the 2S albumin Sin a 1 (UniProt accession P15322) (Figure E2B). MS of purified Act d 12 yielded a heterogeneous profile with two main peaks at 50207.3 and 52280.5 Da (Figure 3C), which might correspond to several isoforms as reported for other 11S globulin counterparts.²⁵ For Act d 13 a single peak at 11359.0 Da was obtained (Figure 3C). The circular dichroism (CD) spectra in the far-UV of purified Act d 12 and Act d 13 demonstrated that both allergens display a structured folding (Figure 3D). Temperature-dependent unfolding experiments (from 20°C to 80 °C) of Act d 12 and Act d 13 showed slight and reversible changes of secondary structure, indicating that both allergens are highly stable to thermal denaturation (Figure 3D).

IgE-binding capacity and *in vivo* allergenicity of Act d 12 and Act d 13 from kiwi seeds

The IgE-binding capacity of purified Act d 12 and Act d 13 from kiwi seeds was assayed by ELISA and immunoblotting (Figure 4). Thirty-nine out of the 55 patients (70.1 %) were positive to purified Act d 12 in immunoblotting (21/28, 75.0 % from group 1 and 18/27, 66.7 % from group 2) (Figure 4A), whereas 10/55 (18.2 %) were positive to Act d 13 in immunoblotting (4/28, 14.3 % from group 1 and 6/27, 22.2 % from group 2) (Figure 4B). Similar ELISA results were observed. Act d 12 was recognized in ELISA by 34/55 (63.6 %) of the patients (19/28 and 15/27, from groups 1 and 2, respectively) (Figure 4A). In contrast, Act d 13 was recognized by 14/55 (25.5 %) of the patients (4/28 and 10/27 from groups 1 and 2, respectively).

The *in vivo* allergenic capacity of purified Act d 12 and Act d 13 from kiwi seeds was demonstrated in kiwifruit allergic patients by SPT (Figure 5). We tested by SPT 4 kiwifruit allergic patients and 3 non-allergic subjects with kiwi seeds, purified Act d 12 and purified Act d 13 and compared the induced wheal areas (Figure 5A). Kiwifruit allergic patients but not healthy controls had positive SPT to kiwi seeds extract (4/4), purified Act d 12 (4/4) and purified Act d 13 (3/4). Kiwifruit allergic patients and non-allergic subjects had comparable responses to histamine as positive control. A representative picture of the forearm from a kiwifruit allergic patient with positive SPT after challenge with the indicated allergens is shown in Figure 5B.

Act d 12 and Act d 13 from kiwi seeds show *in vitro* IgE cross-reactivity with homologous counterparts from peanut and tree nuts

Twenty-four out of the 55 patients included in this study (43.6 %) were also allergic to tree nuts or peanut and 22 out of these 24 patients (91.6 %) were sensitized to Act d 12 or Act d 13 (Table I and Figure 4). We performed *in vitro* IgE-inhibition experiments in

immunoblotting using purified Act d 12 and Act d 13 as well as allergenic extracts from kiwi seeds, peanut, almond, hazelnut and walnut. We pooled the sera from kiwifruit allergic patients sensitized to peanut and tree nuts into two groups (Table II): i) patients with specific IgE to Act d 12, and ii) patients with specific IgE to Act d 13. The IgE reactivity to the protein of 51 kDa in kiwi seeds extract was totally abolished when the pool of sera from patients sensitized to Act d 12 was preadsorbed to kiwi seeds extract or to the purified Act d 12 (Figure 6A). The IgE-binding to purified Act d 12 was significantly inhibited by peanut, almond, hazelnut and walnut (86%, 67%, 66% and 65%, respectively, as determined by scanning densitometry) and completely inhibited by kiwi seeds extract or purified Act d 12 (Figure 6B). The IgE-reactivity to the protein of 12 kDa in kiwi seeds extract was also completely inhibited when the pool of sera from patients sensitized to Act d 13 was preadsorbed to kiwi seeds extract or to the purified Act d 13 (Figure 6C). The inhibition of the IgE-binding to the purified Act d 13 was observed when walnut (85%), peanut (70%) and almond (49%) but not hazelnut (10%) were employed as inhibitors (Figure 6D). Complete inhibition was obtained with kiwi seeds or the purified Act d 13.

DISCUSSION

In this study, we uncovered two novel relevant allergens from kiwi seeds, an 11S globulin (Act d 12) and a 2S albumin (Act d 13) that might have been masked in the context of kiwifruit allergy due to their specific location in seeds. The identification of Act d 12 and Act d 13 from kiwi seeds and their possible availability as purified molecules might well contribute to improve the diagnostic sensitivity, which is currently one of the main drawbacks associated to kiwifruit allergy. Both allergens retained the capacity to bind serum IgE from kiwifruit allergic patients, being Act d 12 recognized by 70.9 % and Act d 13 by 18.2 % of the patients included in this study. Act d 12 and Act d 13 showed *in vivo* allergenicity, as determined by SPT and displayed *in vitro* IgE-cross reactivity with homologous counterparts from peanut and tree nuts.

Since the first case of kiwifruit allergy was reported in 1981,²⁶ the prevalence of this IgE-mediated food allergy has enormously increased.¹⁻⁴ Kiwifruit allergy is frequently associated to pollen or latex allergy with a wide range of clinical symptoms, which compounds accurate diagnosis.⁵ Diagnosis of kiwifruit allergy has significantly improved during the last years.^{1, 2, 5, 14} However, the main drawbacks are the low specificity for prick to prick tests, likely due to the analysis of dichotomized instead of the size of SPT outcomes,²⁻⁴ and the low sensitivity for *in vivo* SPT and *in vitro* serum specific-IgE determinations. Low sensitivity has been attributed to the lack of relevant allergens in commercial kiwifruit extracts³⁻⁵ and to the different content of allergenic proteins depending on the kiwi varieties.²⁷⁻³⁰ In this study, we included 55 patients with a suggestive clinical history of kiwifruit allergy reporting classical immediate IgE-mediated symptoms after ingestion of kiwifruit. Only 50.9 % of the patients showed positive SPT with commercial kiwifruit extract and 34.5 % of the patients were positive in ELISA, which is in accordance with the low sensitivity previously reported for such determinations.^{1, 5, 14} A recent study showed that the combination of purified kiwifruit allergens (Act d 1, 2, 5, 6, 9 y

10) improved the *in vitro* diagnostic sensitivity up to 65%.¹ This study also suggested that 35 % of kiwifruit allergic patients might be sensitized to undiscovered relevant allergens. Considering that all the currently known kiwifruit allergens, except Act d 10 (LTP),^{5, 15} have been exclusively described in the pulp of kiwifruit together with the fact that seeds are commonly ingested with the pulp, we hypothesized that novel key allergens in kiwi seeds might have been masked in the context of kiwifruit allergy.

To identify novel allergens in kiwi seeds we grouped the patients according to positive or negative SPT to kiwifruit extract (groups 1 and 2, respectively) and tested the sera in immunoblotting with home-made kiwifruit extracts from total (pulp and seeds), pulp and seeds prepared following protocols previously described for each specific tissue (see **Online Repository**).^{31, 32} We detected two IgE-reactive protein bands of 50 and 12 kDa in kiwi seeds but not in total or pulp kiwifruit extracts, which were identified by proteomic tools and Edman degradation sequencing as an 11S globulin (Act d 12) and a 2S albumin (Act d 13), respectively. These allergens from kiwi seeds were not detected in total and pulp kiwifruit extracts, likely due to the poor extraction of such proteins when using conventional protocols to prepare aqueous extracts from fruits.^{31, 32} The lack of kiwi seeds allergens in commercially available kiwifruit protein extracts might well justify why patients from group 2 in this study did not show positive SPT. Around 85 % of the patients included in this study were positive to purified Act d 12 or Act d 13 from kiwi seeds, whereas only 45.5 % of the patients showed positive responses to total or pulp kiwifruit extracts in immunoblotting.

The use of well-defined purified allergens is very useful to improve diagnosis and treatment of allergic diseases.³³⁻³⁵ The structural characterization of purified 11S globulin Act d 12 and 2S albumin Act d 13 demonstrated that both purified allergens display classical features of members from these protein families. 11S globulins and 2S albumins are very stable seed-specific storage proteins composed of two polypeptide chains linked by disulphide bridges that have been previously described as potent food allergens able to

induce primary sensitization at the gastrointestinal level and involved in IgE cross-reactivity.^{21, 22, 31, 36-40} Act d 12 represents a major allergen recognized by 70.9 % of the kiwifruit allergic patients in this study. Five patients (# 20 and 25 from group 1 and 30, 42 and 44 from group 2) that were positive to purified Act d 12 in immunoblotting did not react to the purified protein in ELISA, suggesting the existence of internal epitopes that are exposed after SDS-PAGE. Act d 13 is a minor allergen being recognized by 18.2% of the patients and 4 patients (# 30, 34, 41 and 42 from group 2) reacted to purified Act d 13 in ELISA but not in immunoblotting, suggesting the existence of potential conformational epitopes in the allergen that are altered under the denaturing conditions. Importantly, both native allergens demonstrated *in vivo* allergenicity as they were able to induce IgE crosslinking in effector skin mast cells when assayed in SPT. *In vitro* IgE-inhibition experiments demonstrated that purified Act d 12 and Act d 13 conserved common epitopes to homologous counterparts from peanut, almond, hazelnut or walnut, suggesting that Act d 12 and Act d 13 might be also involved in cross-reactivity with these allergenic sources. Although 91.6 % (22/24) of the kiwifruit allergic patients of this study that were also allergic to tree nuts or peanut were sensitized to Act d 12 or Act d 13 (15/24 to Act d 12 and 9/24 to Act d 13), further detailed studies are required to elucidate the potential clinical relevance of this observed *in vitro* IgE-cross reactivity.

Collectively, we identified and deeply characterized two novel relevant allergens from kiwi seeds. The inclusion of purified Act d 12 and Act d 13 in CRD might well contribute to significantly increase the diagnostic sensitivity of kiwifruit allergy, which might be especially relevant to properly diagnose those patients showing negative SPT with commercial kiwifruit extracts. Although our results indicate that Act d 12 and Act d 13 show *in vitro* IgE cross-reactivity with homologous counterparts from tree nuts and peanut, further detailed studies are required to firmly confirm the clinical relevance of this fact.

REFERENCES

1. Le TM, Bublin M, Breiteneder H, Fernandez-Rivas M, Asero R, Ballmer-Weber B, et al. Kiwifruit allergy across Europe: clinical manifestation and IgE recognition patterns to kiwifruit allergens. *J Allergy Clin Immunol* 2013; 131:164-71.
2. Bublin M, Pfister M, Radauer C, Oberhuber C, Bulley S, Dewitt AM, et al. Component-resolved diagnosis of kiwifruit allergy with purified natural and recombinant kiwifruit allergens. *J Allergy Clin Immunol* 2010; 125:687-94, 94 e1.
3. Aleman A, Sastre J, Quirce S, de las Heras M, Carnes J, Fernandez-Caldas E, et al. Allergy to kiwi: a double-blind, placebo-controlled food challenge study in patients from a birch-free area. *J Allergy Clin Immunol* 2004; 113:543-50.
4. Lucas JS, Grimshaw KE, Collins K, Warner JO, Hourihane JO. Kiwi fruit is a significant allergen and is associated with differing patterns of reactivity in children and adults. *Clin Exp Allergy* 2004; 34:1115-21.
5. Bublin M. Kiwifruit allergies. *Adv Food Nutr Res* 2013; 68:321-40.
6. Pastorello EA, Pravettoni V, Ispano M, Farioli L, Ansaloni R, Rotondo F, et al. Identification of the allergenic components of kiwi fruit and evaluation of their cross-reactivity with timothy and birch pollens. *J Allergy Clin Immunol* 1996; 98:601-10.
7. Kerzl R, Simonowa A, Ring J, Ollert M, Mempel M. Life-threatening anaphylaxis to kiwi fruit: protective sublingual allergen immunotherapy effect persists even after discontinuation. *J Allergy Clin Immunol* 2007; 119:507-8.
8. Mempel M, Rakoski J, Ring J, Ollert M. Severe anaphylaxis to kiwi fruit: Immunologic changes related to successful sublingual allergen immunotherapy. *J Allergy Clin Immunol* 2003; 111:1406-9.
9. Brehler R, Theissen U, Mohr C, Luger T. "Latex-fruit syndrome": frequency of cross-reacting IgE antibodies. *Allergy* 1997; 52:404-10.

- 358 10. Diaz-Perales A, Collada C, Blanco C, Sanchez-Monge R, Carrillo T, Aragoncillo C,
 359 et al. Cross-reactions in the latex-fruit syndrome: A relevant role of chitinases but
 360 not of complex asparagine-linked glycans. *J Allergy Clin Immunol* 1999; 104:681-7.
- 361 11. Palacin A, Rodriguez J, Blanco C, Lopez-Torrejon G, Sanchez-Monge R, Varela J,
 362 et al. Immunoglobulin E recognition patterns to purified Kiwifruit (*Actinidia*
 363 *deliciosa*) allergens in patients sensitized to Kiwi with different clinical symptoms.
 364 *Clin Exp Allergy* 2008; 38:1220-8.
- 365 12. Burks AW, Tang M, Sicherer S, Muraro A, Eigenmann PA, Ebisawa M, et al. ICON:
 366 food allergy. *J Allergy Clin Immunol* 2012; 129:906-20.
- 367 13. Sampson HA, Gerth van Wijk R, Bindslev-Jensen C, Sicherer S, Teuber SS, Burks
 368 AW, et al. Standardizing double-blind, placebo-controlled oral food challenges:
 369 American Academy of Allergy, Asthma & Immunology-European Academy of
 370 Allergy and Clinical Immunology PRACTALL consensus report. *J Allergy Clin*
 371 *Immunol* 2012; 130:1260-74.
- 372 14. Bublin M, Dennstedt S, Buchegger M, Antonietta Ciardiello M, Bernardi ML, Tuppo
 373 L, et al. The performance of a component-based allergen microarray for the
 374 diagnosis of kiwifruit allergy. *Clin Exp Allergy* 2011; 41:129-36.
- 375 15. Bernardi ML, Giangrieco I, Camardella L, Ferrara R, Palazzo P, Panico MR, et al.
 376 Allergenic lipid transfer proteins from plant-derived foods do not immunologically
 377 and clinically behave homogeneously: the kiwifruit LTP as a model. *PLoS One*
 378 2011; 6:e27856.
- 379 16. Vereda A, Sirvent S, Villalba M, Rodriguez R, Cuesta-Herranz J, Palomares O.
 380 Improvement of mustard (*Sinapis alba*) allergy diagnosis and management by
 381 linking clinical features and component-resolved approaches. *J Allergy Clin*
 382 *Immunol* 2011; 127:1304-7.

- 383 17. Alcocer M, Rundqvist L, Larsson G. Ber e 1 protein: the versatile major allergen
384 from Brazil nut seeds. *Biotechnol Lett* 2012; 34:597-610.
- 385 18. Dalal I, Goldberg M, Katz Y. Sesame seed food allergy. *Curr Allergy Asthma Rep*
386 2012; 12:339-45.
- 387 19. Vereda A, van Hage M, Ahlstedt S, Ibanez MD, Cuesta-Herranz J, van Odijk J, et
388 al. Peanut allergy: Clinical and immunologic differences among patients from 3
389 different geographic regions. *J Allergy Clin Immunol* 2011; 127:603-7.
- 390 20. Radauer C, Breiteneder H. Evolutionary biology of plant food allergens. *J Allergy*
391 *Clin Immunol* 2007; 120:518-25.
- 392 21. Sirvent S, Akotenou M, Cuesta-Herranz J, Vereda A, Rodriguez R, Villalba M, et al.
393 The 11S globulin Sin a 2 from yellow mustard seeds shows IgE cross-reactivity
394 with homologous counterparts from tree nuts and peanut. *Clin Transl Allergy* 2012;
395 2:23.
- 396 22. Sirvent S, Palomares O, Cuesta-Herranz J, Villalba M, Rodriguez R. Analysis of
397 the Structural and Immunological Stability of 2S Albumin, Nonspecific Lipid
398 Transfer Protein, and Profilin Allergens from Mustard Seeds. *J Agric Food Chem*
399 2012.
- 400 23. Wood RA. Oral Food Challenge Testing. In: Atkinson F BB, Busse W, Holgate S,
401 Lemanske R, Simons FER editor, editor. *Middleton's Allergy: Principles and*
402 *Practice*. 7th ed. New York, NY: Mosby; 2009. p. 1309-17.
- 403 24. Cuesta-Herranz J, Barber D, Blanco C, Cistero-Bahima A, Crespo JF, Fernandez-
404 Rivas M, et al. Differences among pollen-allergic patients with and without plant
405 food allergy. *Int Arch Allergy Immunol* 2010; 153:182-92.
- 406 25. Palomares O, Vereda A, Cuesta-Herranz J, Villalba M, Rodriguez R. Cloning,
407 sequencing, and recombinant production of Sin a 2, an allergenic 11S globulin from
408 yellow mustard seeds. *J Allergy Clin Immunol* 2007; 119:1189-96.

- 409 26. Fine AJ. Hypersensitivity reaction to kiwi fruit (Chinese gooseberry, *Actinidia*
410 *chinensis*). *J Allergy Clin Immunol* 1981; 68:235-7.
- 411 27. Le TM, Fritsche P, Bublin M, Oberhuber C, Bulley S, van Hoffen E, et al.
412 Differences in the allergenicity of 6 different kiwifruit cultivars analyzed by prick-to-
413 prick testing, open food challenges, and ELISA. *J Allergy Clin Immunol* 2011;
414 127:677-9 e1-2.
- 415 28. Bublin M, Mari A, Ebner C, Knulst A, Scheiner O, Hoffmann-Sommergruber K, et
416 al. IgE sensitization profiles toward green and gold kiwifruits differ among patients
417 allergic to kiwifruit from 3 European countries. *J Allergy Clin Immunol* 2004;
418 114:1169-75.
- 419 29. Chen L, Lucas JS, Hourihane JO, Lindemann J, Taylor SL, Goodman RE.
420 Evaluation of IgE binding to proteins of hardy (*Actinidia arguta*), gold (*Actinidia*
421 *chinensis*) and green (*Actinidia deliciosa*) kiwifruits and processed hardy kiwifruit
422 concentrate, using sera of individuals with food allergies to green kiwifruit. *Food*
423 *Chem Toxicol* 2006; 44:1100-7.
- 424 30. Maddumage R, Nieuwenhuizen NJ, Bulley SM, Cooney JM, Green SA, Atkinson
425 RG. Diversity and relative levels of actinidin, kiwellin, and thaumatin-like allergens
426 in 15 varieties of kiwifruit (*Actinidia*). *J Agric Food Chem* 2013; 61:728-39.
- 427 31. Palomares O, Cuesta-Herranz J, Vereda A, Sirvent S, Villalba M, Rodriguez R.
428 Isolation and identification of an 11S globulin as a new major allergen in mustard
429 seeds. *Ann Allergy Asthma Immunol* 2005; 94:586-92.
- 430 32. Palomares O, Villalba M, Quiralte J, Polo F, Rodriguez R. 1,3-beta-glucanases as
431 candidates in latex-pollen-vegetable food cross-reactivity. *Clin Exp Allergy* 2005;
432 35:345-51.

- 433 33. Palomares O, Alcantara M, Quiralte J, Villalba M, Garzon F, Rodriguez R. Airway
434 disease and thaumatin-like protein in an olive-oil mill worker. *N Engl J Med* 2008;
435 358:1306-8.
- 436 34. Hiller R, Laffer S, Harwanegg C, Huber M, Schmidt WM, Twardosz A, et al.
437 Microarrayed allergen molecules: diagnostic gatekeepers for allergy treatment.
438 *FASEB J* 2002; 16:414-6.
- 439 35. Valenta R, Twaroch T, Swoboda I. Component-resolved diagnosis to optimize
440 allergen-specific immunotherapy in the Mediterranean area. *J Investig Allergol Clin*
441 *Immunol* 2007; 17 Suppl 1:36-40.
- 442 36. Palomares O, Monsalve RI, Rodriguez R, Villalba M. Recombinant pronapin
443 precursor produced in *Pichia pastoris* displays structural and immunologic
444 equivalent properties to its mature product isolated from rapeseed. *Eur J Biochem*
445 2002; 269:2538-45.
- 446 37. Menendez-Arias L, Moneo I, Dominguez J, Rodriguez R. Primary structure of the
447 major allergen of yellow mustard (*Sinapis alba* L.) seed, Sin a I. *Eur J Biochem*
448 1988; 177:159-66.
- 449 38. Palomares O, Cuesta-Herranz J, Rodriguez R, Villalba M. A recombinant precursor
450 of the mustard allergen Sin a 1 retains the biochemical and immunological features
451 of the heterodimeric native protein. *Int Arch Allergy Immunol* 2005; 137:18-26.
- 452 39. Beyer K, Bardina L, Grishina G, Sampson HA. Identification of sesame seed
453 allergens by 2-dimensional proteomics and Edman sequencing: seed storage
454 proteins as common food allergens. *J Allergy Clin Immunol* 2002; 110:154-9.
- 455 40. Beyer K, Grishina G, Bardina L, Grishin A, Sampson HA. Identification of an 11S
456 globulin as a major hazelnut food allergen in hazelnut-induced systemic reactions.
457 *J Allergy Clin Immunol* 2002; 110:517-23.

458 Table I. Clinical characteristics of patients with kiwifruit allergy

GROUP 1: patients with positive SPT to kiwifruit							GROUP 2: patients with negative SPT to kiwifruit						
Patient no.	Sex/Age (y)	Kiwifruit Symptoms	Kiwifruit extract		Other food allergies	Pollen allergy	Patient no.	Sex/Age (y)	Kiwifruit Symptoms	Kiwifruit extract		Other food allergies	Pollen allergy
			SPT*	ELISA†						SPT*	ELISA†		
1	F/32	AE	22	0.199	r,m,f,tr	Yes	29	F/29	OAS	Neg	0.051	s	No
2	M/27	U	22	0.146	r,n,p	Yes	30	M/30	AE	Neg	0.109	No	Yes
3	F/23	AE	44	0.075	r,pn,w,av,tr,b,t	Yes	31	F/25	CU	Neg	0.108	pt	No
4	M/47	OAS	22	0.056	m,pn	No	32	F/46	OAS	Neg	0.084	tr	No
5	F/32	U/OAS	22	0.808	No	Yes	33	M/20	OAS	Neg	0.067	No	No
6	M/48	AN	44	0.140	No	Yes	34	F/32	AN	Neg	0.075	No	No
7	F/25	AN	22	0.058	r,m	No	35	M/17	CU	Neg	0.085	n	Yes
8	F/42	OAS	22	0.064	r	Yes	36	F/25	OAS	Neg	0.088	n,b,pn	Yes
9	F/18	AE/OAS	22	0.115	n	No	37	F/53	AE	Neg	0.092	gr,p	No
10	M/21	AN	44	0.177	r,m,pn	No	38	F/75	OAS	Neg	0.105	r	No
11	M/77	OAS	22	0.113	No	No	39	F/36	OAS	Neg	0.112	r,n,p,tr	No
12	M/19	OAS	12	0.075	n,b,gr,p	No	40	F/33	OAS	Neg	0.079	r,n,p,t,s	Yes
13	F/26	OAS	22	0.068	r,n,t,p	Yes	41	M/33	OAS	Neg	0.080	r	Yes
14	M/51	GD	12	0.154	n,p	No	42	F/50	AN	Neg	0.081	No	Yes
15	F/20	OAS	12	0.063	r,n,p	Yes	43	M/37	OAS	Neg	0.235	r,n,p,m,f	Yes
16	M/30	AN	9	0.086	No	Yes	44	F/37	OAS	Neg	0.001	r,b,m,t,f,pn,tr	No
17	F/46	OAS	16	0.086	r,n,p,m,f,t,w	No	45	F/35	OAS	Neg	0.009	r,pn,t	Yes
18	M/19	AE	30	0.441	r,n,p,b,m,w,pn,f,gr	Yes	46	F/75	OAS	Neg	0.006	m,pn,gr,f	No
19	F/16	AE	16	0.171	r,n,tr,pn	Yes	47	F/21	OAS	Neg	0.020	r,n,p,b,t,pn,gr	Yes
20	F/43	OAS	40	0.824	r,n	Yes	48	F/53	OAS	Neg	0.013	r,m,pn,gr,tr	Yes
21	F/26	AN	25	0.376	No	No	49	F/28	OAS	Neg	0.020	n,av,m,pn	Yes
22	M/39	U	12	0.069	r,n,p	Yes	50	F/29	OAS	Neg	0.009	av,m,w,tr	Yes
23	F/34	AE	16	0.056	r,n,p,m,w,tr	No	51	F/24	OAS	Neg	0.007	r,b,m,pn	Yes
24	F/19	OAS	9	0.066	r,n,p	Yes	52	F/72	OAS	Neg	0.000	r,pn,tr,gr	Yes
25	M/36	AN	35	0.110	r,n,p,m,w,tr,av,gr,s	Yes	53	F/34	OAS	Neg	0.173	No	No
26	F/31	AN	35	0.226	No	No	54	F/39	OAS	Neg	0.142	n,p	Yes
27	F/34	OAS	16	0.118	No	Yes	55	F/32	OAS	Neg	0.219	r	No
28	F/17	OAS	12	0.212	r,n	Yes							

y: year; M/F: male/female; Neg: negative; AE: angioedema; AN: anaphylaxis; CU: contact urticaria; GD: gastrointestinal disorders; OAS: oral allergy syndrome; U: urticaria; av: avocado; b: banana; f: fig; gr: grape; m: melon; n: tree nuts including almond; p: peanut; pn: pineapple; pt: potato; r: *rosaceae* excluding almond; s: soy; t: tomato; tr: tropical; w: watermelon;

* Skin prick test wheal area in mm²

† Specific IgE determined in ELISA as OD at 492 nm

Table II. Clinical features of the patients selected for *in vitro* IgE inhibition experiments

Sera pool	Patient number	Kiwi symptoms	Tree nuts* symptoms	SPT (mm ²)				
				Kiwi	Peanut	Almond	Hazelnut	Walnut
Act d 12 positive	13	OAS	OAS	22	12	12	12	Neg
	22	U	A, AN	12	12	Neg	48	Neg
	24	OAS	OAS	9	9	20	12	9
	47	OAS	OAS	Neg	18	35	15	42
	54	OAS	OAS	Neg	ND	ND	ND	ND
Act d 13 positive	2	U	OAS	22	Neg	Neg	22	Neg
	15	OAS	OAS	12	12	Neg	10	12
	28	OAS	OAS	12	ND	ND	ND	ND
	39	OAS	OAS	Neg	12	Neg	22	22
	43	OAS	AN, OAS	Neg	22	22	22	Neg

Neg: negative; A: asthma; AE: angioedema; AN: anaphylaxis; OAS: oral allergy syndrome; U: Urticaria; ND: not done
 *including almond

LEGEND TO FIGURES

FIG 1. SDS-PAGE for total kiwi (left), kiwi pulp (middle) and kiwi seeds (right) extracts and Coomassie Blue staining (CBS) or IgE reactivity of sera from patients allergic to kiwi (group 1, patients with positive SPT to kiwi extract) and (group 2, patients with negative SPT to kiwi extract).

FIG 2. IgE-reactivity of sera from patients allergic to kiwifruit of group 1 and 2 (lanes 1 to 55) to kiwi seeds extract. C1 and C2 are sera from non-atopic and pollen allergic subjects, respectively.

FIG 3. Identification and structural characterization of purified 11S globulin (Act d 12) and 2S albumin (Act d 13). **(A)** MS-profile of the indicated proteins. Inset, SDS-PAGE of kiwi seeds extract stained with Coomassie blue staining (CBS) to visualize the excised bands (indicated arrows). Identified peptides are shown. The shadow arrows indicate the peptides identified by MS/MS analysis. **(B)** SDS-PAGE and CBS of kiwi seeds extract, purified Act d 12 and purified Act d 13 in the absence or presence of the reducing agent, β -mercaptoethanol (β me). **(C)** Molecular mass determination of the purified Act d 12 and Act d 13 by MS. **(D)** CD spectra of purified Act d 12 and Act d 13 in the far-UV at 20 °C, at 85° C and cooling down again at 20 °C. Secondary structure contributions (α H, α -helix; β S, β -sheet; β T, β -turn; RC, random coil) are shown.

FIG 4. IgE-reactivity of purified Act d 12 **(A)** and Act d 13 **(B)**. ELISA (*upper panel*) and immunoblotting (*lower panel*) are shown for the sera of the 55 patients allergic to kiwifruit grouped into patients with positive or negative SPT to kiwifruit extract. OD, optical density. C1 and C2, sera from non-atopic and pollen allergic subject, respectively.

FIG 5. (A) Induced wheal size in kiwifruit allergic patients (A) and non-allergic controls (NA) by SPT with kiwi seeds extract, purified Act d 12, purified Act d 13 and histamine (positive control). Horizontal discontinuous line shows the cut off for negative (PBS) controls. Each symbol represents a single patient or healthy subject **(B)** Representative

486 example of the forearm of a kiwifruit allergic patient with positive SPT to the indicated
487 allergens.

488 **FIG 6.** Immunoblotting inhibition experiments. Inhibition of the IgE-binding to Act d 12
489 contained in kiwi seeds extract **(A)** or to purified Act d 12 **(B)** by the indicated inhibitors
490 when using a pool of sera from kiwifruit allergic patients sensitized to Act d 12. Inhibition of
491 the IgE-binding to Act d 13 contained in kiwi seeds extract **(C)** or to purified Act d 13 **(D)** by
492 the indicated inhibitors when using a pool of sera from kiwifruit allergic patients sensitized
493 to Act d 13. BSA was used as negative control of inhibition. Percentages of inhibition are
494 shown at the bottom of the figures (B) and (D).

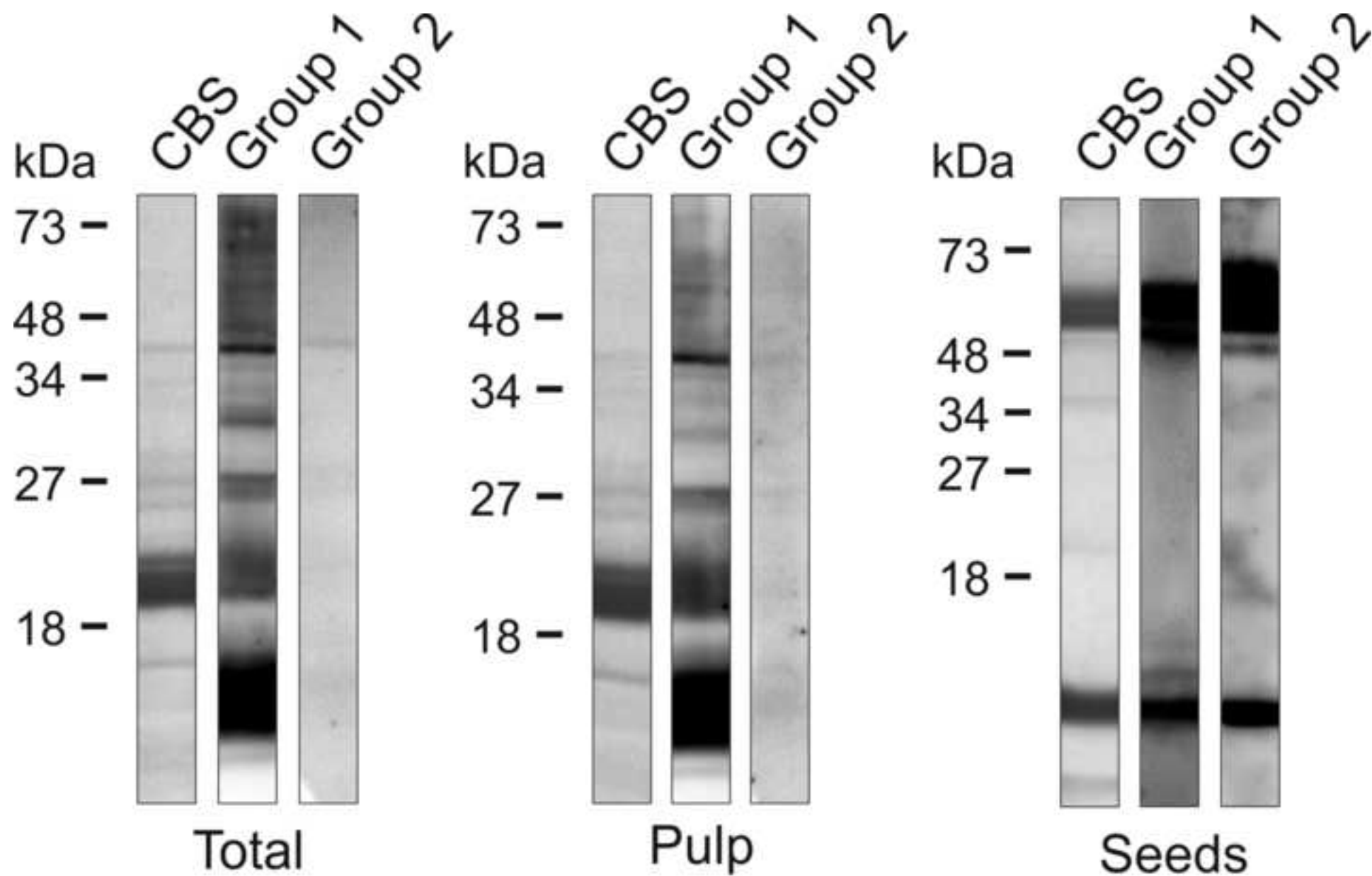


FIGURE 1 Sirvent *et al.*

Figure No. 2
[Click here to download high resolution image](#)

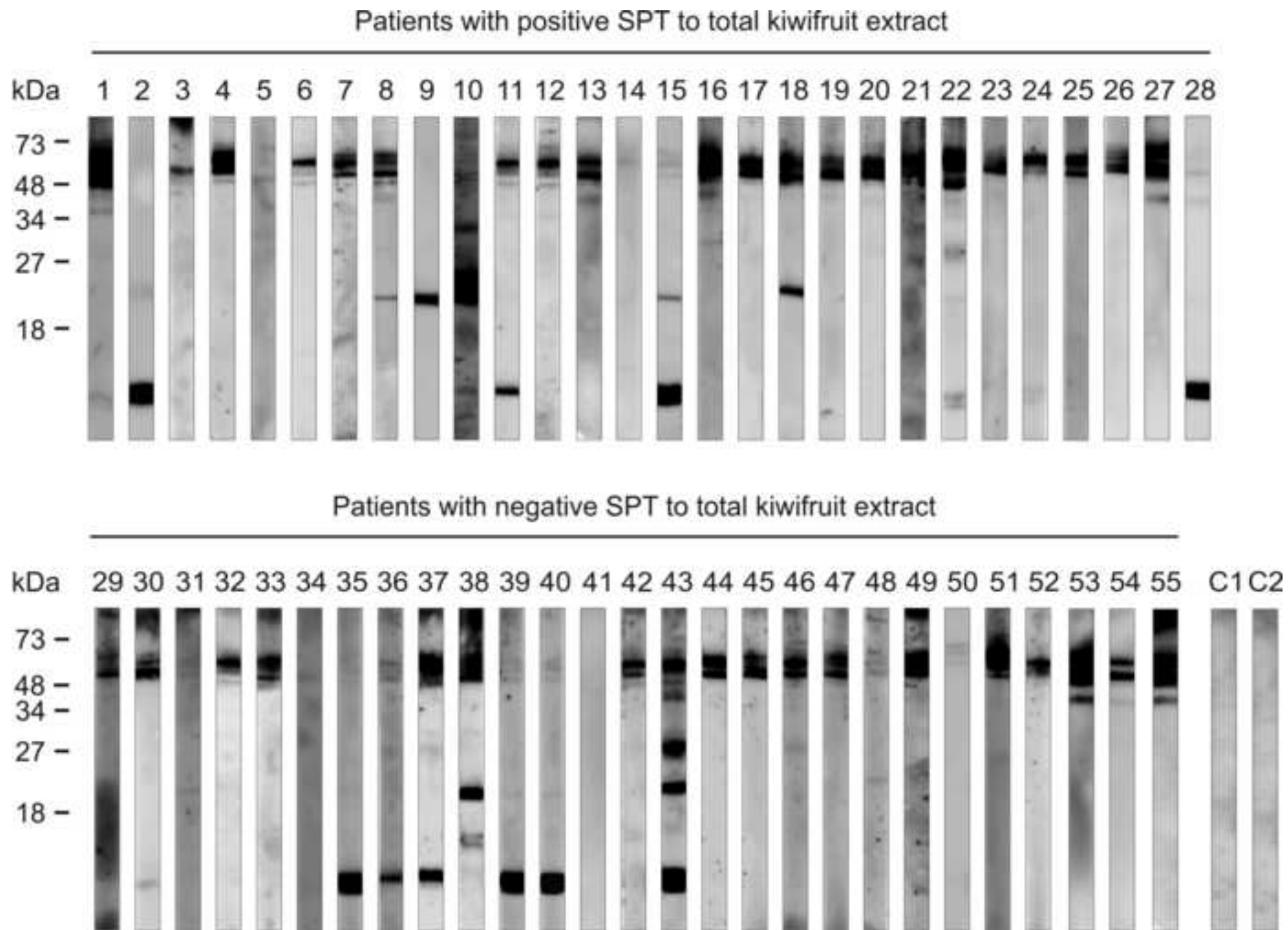


FIGURE 2 Sirvent *et al.*

Figure No. 3
[Click here to download high resolution image](#)

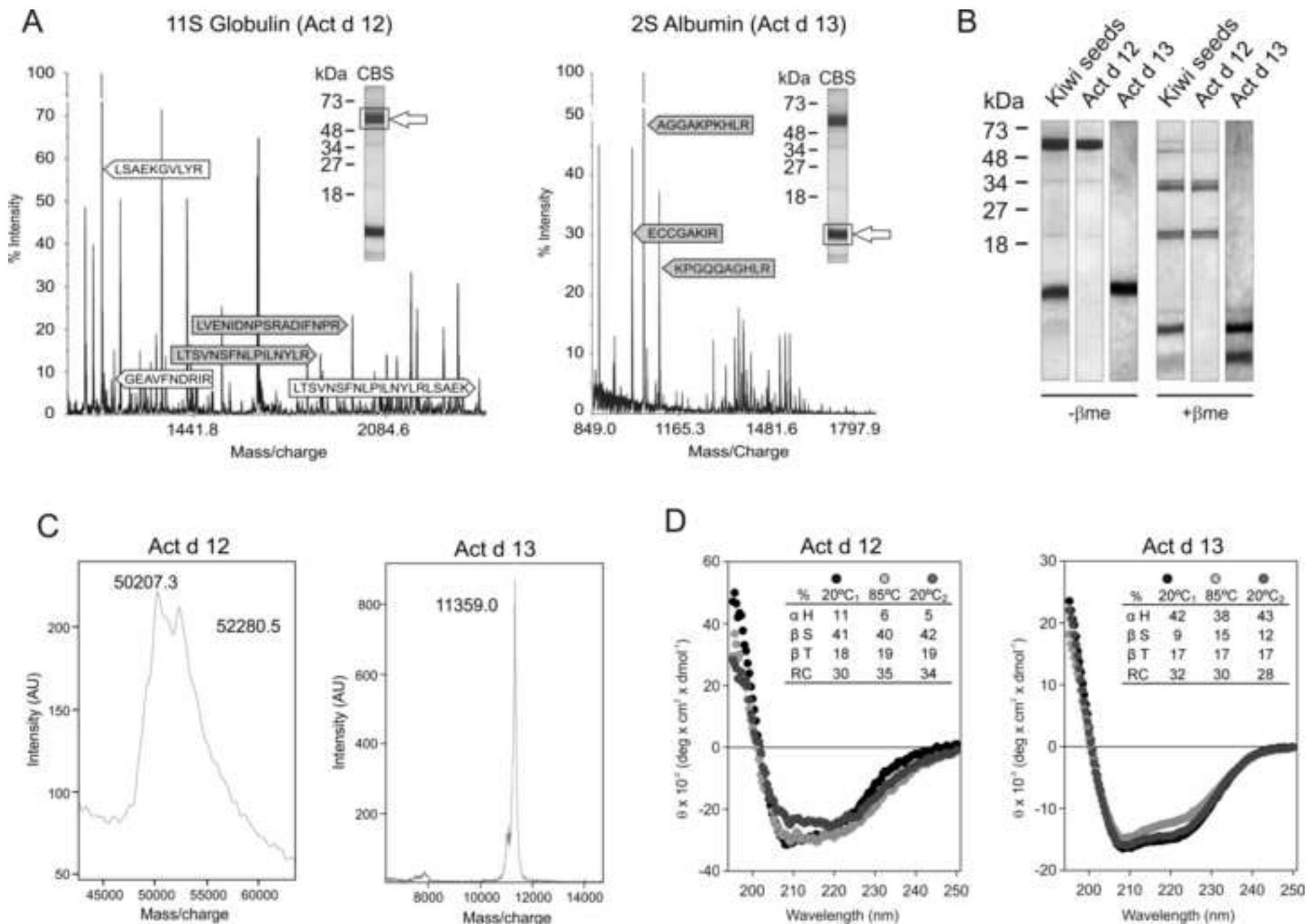


FIGURE 3 Sirvent *et al.*

Figure No. 4
[Click here to download high resolution image](#)

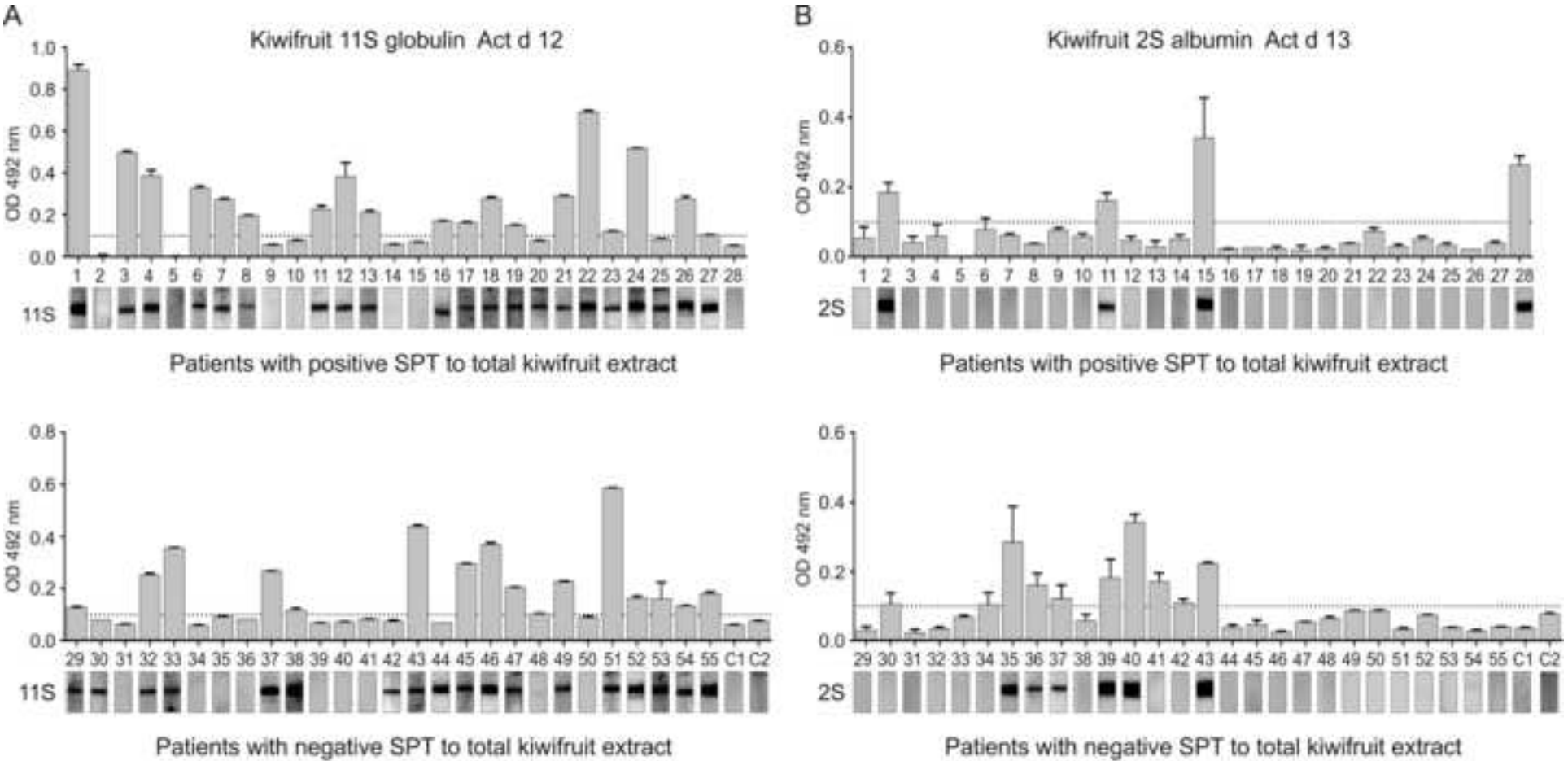


FIGURE 4 Sirvent *et al.*

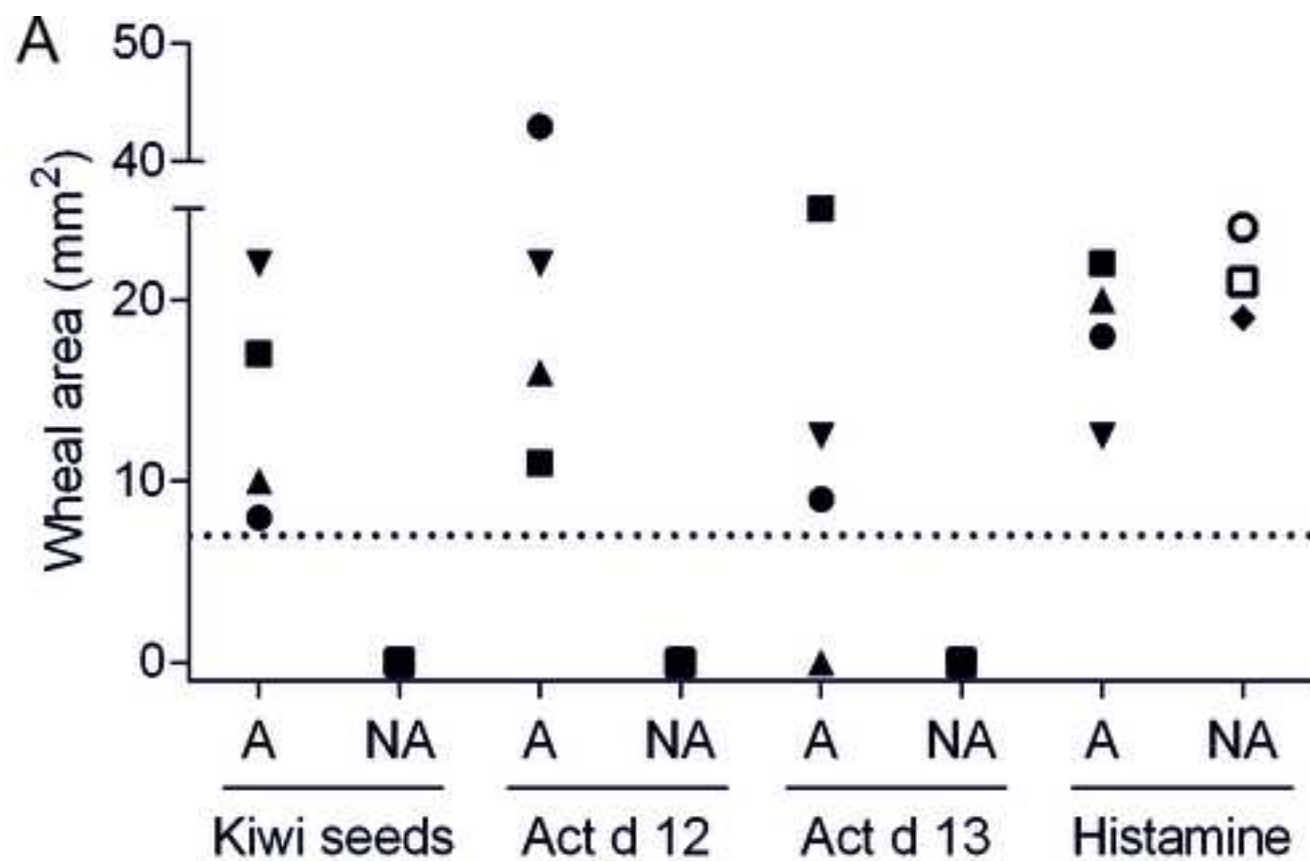


FIGURE 5 Sirvent *et al.*

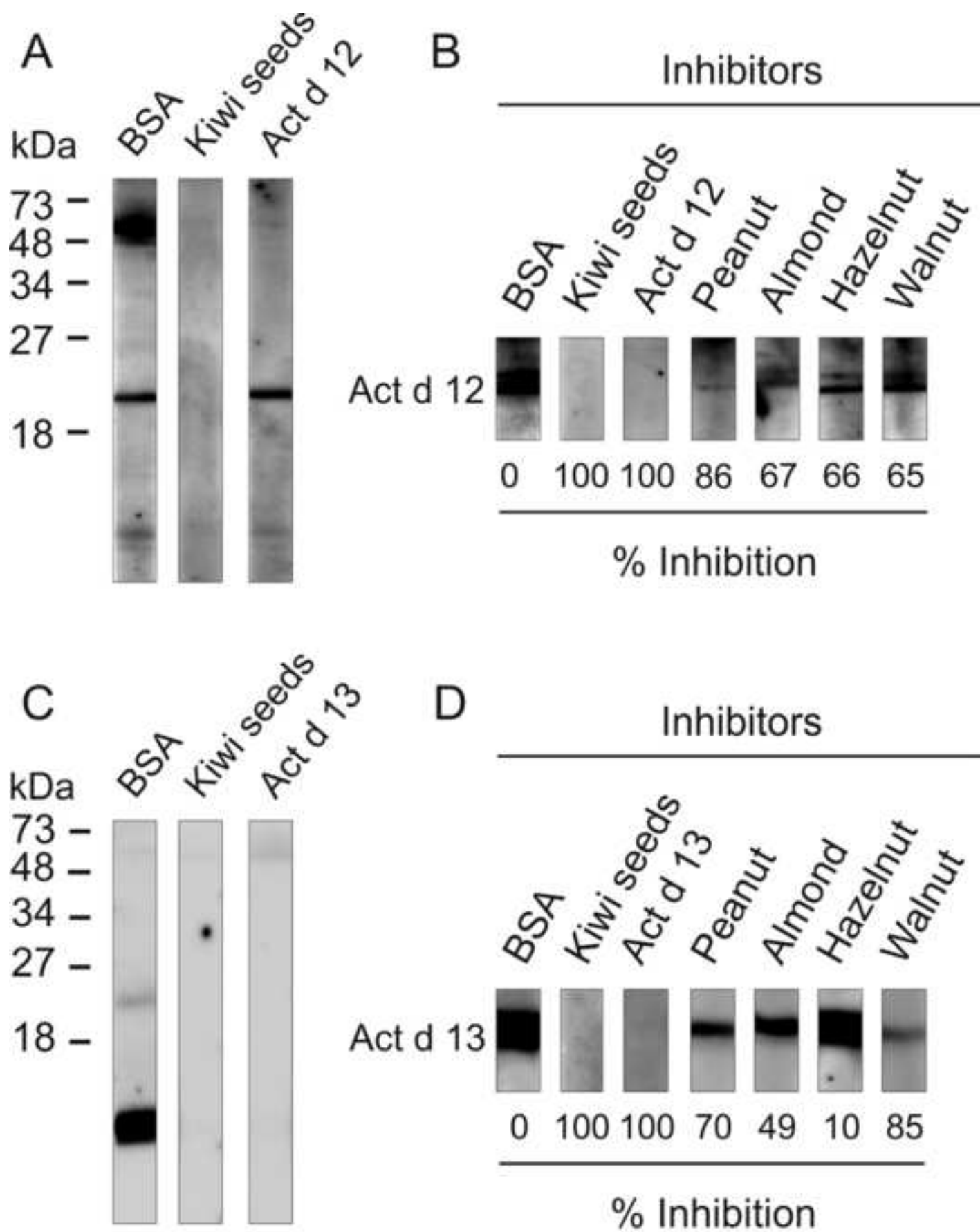


FIGURE 6 Sirvent *et al.*

Online repository (Sirvent et al.)

1 **TITLE PAGE**

2 **Original article**

3 **Unexpected allergenic 11S globulin (Act d 12) and 2S albumin (Act d 13) from kiwi**
4 **seeds as novel masked relevant components in kiwifruit allergy**

5 Sofía Sirvent, PhD,^a Belén Cantó, BSc,^a Javier Cuesta-Herranz, MD,^b Paqui Gómez, MD,^c

6 Natalia Blanca, MD,^d Gabriela Canto, MD,^d Miguel Blanca, MD,^c Rosalía Rodríguez, PhD,^a

7 Mayte Villalba, PhD,^a and Oscar Palomares, PhD^a

8 ^aDepartment of Biochemistry and Molecular Biology, School of Chemistry, Complutense University
9 of Madrid, Spain

10 ^b Allergy Service, Fundación Jiménez Díaz, Madrid, Spain

11 ^c Allergy Service, Hospital Carlos Haya, Málaga, Spain

12 ^d Allergy Service, Hospital Infanta Leonor, Madrid, Spain

13

14

15 **Corresponding Author:** Oscar Palomares, PhD
16 Department of Biochemistry and Molecular Biology,
17 Chemistry School, Complutense University of Madrid,
18 Ciudad Universitaria s/n, 28040 Madrid, Spain.
19 Telephone: + 34 913944161
20 Fax: + 34 913944159
21 Email: oscar.palomares@quim.ucm.es
22

23

24

25 This work was supported by grants: The authors' laboratories are supported by the
26 Spanish Ministerio de Economía y Competitividad (MINECO) SAF2011-26716 and Fondo
27 de Investigación Sanitaria (FIS) RD12/0013/0015. OP is a Ramon y Cajal Scholar funded
28 by MINECO and the European Social Fund.

MATERIALS and METHODS

Preparation of home-made kiwi extracts (total, pulp and seeds)

Kiwi seeds extract was obtained as previously described for yellow mustard seeds¹ with minor modifications. Briefly, kiwi seeds were manually separated from the pulp of kiwifruit (*Actinidia deliciosa*) and lyophilized. Powder of kiwi seeds was suspended in 0.15 M sodium borate buffer, pH 8.0, at a 10% (wt/vol) ratio and gently stirred for 1 hour. After centrifugation at 4°C, the supernatant was collected and the pellet was reextracted twice with the same buffer and conditions. Collected supernatants were lyophilized. The resulting material was then extracted 3 times with 10% (wt/vol) acetone to remove lipid components. The obtained pellets containing extracted proteins were air desiccated and dissolved in 0.15 M ammonium bicarbonate, pH 8. After centrifugation at 8,500 rpm, at 4°C, supernatants were lyophilized, quantified by Lowry method² and stored at –20°C until used. Protein extracts for almond, hazelnut, peanut and walnut were prepared following the same protocol.

Total (pulp and seed) or only pulp Kiwifruit extracts were prepared using as starting material peeled kiwifruit that were homogenized in 10% (wt/vol) ammonium bicarbonate 50 mM and PMSF 1 mM in a blender. After 1 h mixing at 4 °C, the homogenate was centrifuged for 30 min at 8,500 rpm at 4 °C. The pellet was extracted again using the same solvent and the supernatants were collected, lyophilized and stored at -20 °C until used. The lyophilized protein extracts were resuspended in 0.15 M ammonium bicarbonate, pH 8.0 and quantified by Lowry method.²

Skin prick tests

SPT were performed in all patients according to standard procedures.³ The panel of commercial food and pollen extracts included the most relevant allergenic sources in the Mediterranean area. SPT with home-made kiwi seeds extract (100 µg/mL) and with

purified natural Act d 12 (20 µg/mL) and Act d 13 (20 µg/mL) were performed. Histamine dihydrochloride (10 mg/mL) and physiologic saline solutions were used as the positive and negative controls, respectively. Results of SPT were expressed as median wheal size in mm² and a wheal diameter <7 mm² was considered as negative.

Purification of 11S globulin Act d 12 and 2S albumin Act d 13

Lyophilized kiwi seed extract was dissolved in 0.15 M ammonium bicarbonate, pH 8.0, and applied onto a Sephadex G-50 column. Fractions containing protein bands of around 51 and 12 kDa were separately pooled in Batch 1 and Batch 2, respectively. The batch 1 containing the 11S globulin Act d 12 of around 51 kDa was further subjected to ion-exchange chromatography on a DEAE-Cellulose column equilibrated in 20 mM ammonium bicarbonate buffer, pH 8.0. Proteins were eluted with a gradient from 20 to 500 mM ammonium bicarbonate buffer, pH 8.0 and visualized by Coomassie blue staining (CBS) after SDS-PAGE. The fractions containing the 11S globulin Act d 12 were pooled together. The batch 2 containing the 2S albumin Act d 13 of around 12 kDa was resolved on a reverse-phase high-performance liquid chromatographic (RP-HPLC) nucleosil C-18 column with a gradient (25%–45%) of acetonitrile in 0.1% trifluoroacetic acid. SDS-PAGE and CBS were carried out for all purification steps. IgE reactivity of the Act d 12 and Act d 13 was analyzed for each purification step by using a pool of sera from kiwifruit allergic patients diluted 1/3.

Immunoblotting

Immunodetection of allergenic proteins after SDS-PAGE was performed with serum from patients allergic to kiwifruit (diluted 1/3) as described.⁴ The binding of human IgE was detected by mouse anti-human IgE antibodies (diluted 1:5000; ALK-Abelló), followed by horseradish peroxidase-labelled goat anti-mouse IgG (diluted 1:5000; Pierce). The signal

was developed with the ECL-Western blotting reagent (Amersham). For IgE-inhibition experiments the pool of sera (1/3 diluted) was preadsorbed with 1 mg/mL of peanut, almond, hazelnut and walnut protein extracts prepared as previously described for yellow mustard seeds¹ or with 20 µg/mL of purified proteins overnight at 4°C. BSA protein was used as negative control of inhibition. Volummograms of the reactive bands were analyzed by scanning densitometry using Multigauge software (Fujifilm).

ELISA

IgE quantitation was performed by ELISA in microtiter plates (Costar) coated with 100 µl/well of purified proteins (2 µg/mL) or total kiwifruit extract (20 µg/mL).⁴ Plates were incubated with serum from kiwifruit allergic patients (dilution 1/3) and binding of IgE was detected as described above. Peroxidase reaction was developed using fresh enzyme substrate and measuring optical density (OD) at 492 nm. Each value was calculated as the mean of 2 determinations after blank subtraction. OD values <0.1 were considered negative responses.

Analytical procedures

SDS-PAGE was performed in 17 % polyacrylamide gels. Proteins were visualized by Coomassie blue staining (CBS) or alternatively transferred to nitrocellulose membranes (Amersham). The protein concentration was determined using the method of bicinchoninic acid (Pierce Chemical Co).

Molecular mass determination of purified Act d 12 and Act d 13 was performed by mass spectrometry using a Bruker Reflex IV matrix-assisted laser-desorption ionization time-of-flight mass spectrometer (Bruker- Franzen Analytik), as described.⁵ The equipment was externally calibrated employing single-, double- and triple-charged signals from either cytochrome C (12,360 Da) or bovine serum albumin (66,430 Da).

N-terminal Edman degradation of purified Act d 12 and Act d 13 was performed using a 494 sequencer (Applied Biosystems-PE Corp.).

Proteomic procedures

Digestion, MS, and MS/MS analyses of the excised bands of around 51 and 12 kDa from the SDS-PAGE separations of kiwi seeds extract were carried out as previously described.¹ The MS analyses were performed in a Voyager-DESTR instrument (PerSeptive Biosystems). All mass spectra were calibrated externally using a standard peptide mixture (Sigma-Aldrich). MS/MS sequencing analysis were carried out using the MALDI-tandem-TOF MS spectrometer 4700 Proteomics Analyzer (Applied Biosystems).

Circular Dichroism

The CD spectra were obtained using a JASCO J-715 spectropolarimeter (Japan Spectroscopic Co.) fitted with a 150 W xenon lamp and connected to a Nestlab RTE-111 thermostabilizer bath, at 20 °C and 85 °C, as described.⁶ Far-UV spectra (190–250 nm wavelength) were registered using optical-path cell of 0.1 cm. The protein concentration was 0.2 mg/mL in 20 mM ammonium bicarbonate, pH 8. Mean residue mass ellipticities were calculated based on 110 as the average molecular mass/residue and expressed in terms of θ (degree \times cm² \times dmol⁻¹). Final spectra were corrected by subtracting the corresponding baseline spectrum obtained for the buffer alone under identical conditions.

RESULTS

Purification of 11S globulin Act d 12 and 2S albumin Act d 13 from kiwi seeds

Lyophilized kiwifruit seeds protein extract was separated by size in a gel-filtration chromatography (Figure E3A). Fractions containing the proteins of around 51 kDa (11S globulin Act d 12) and 12 kDa (2S albumin Act d 13) were pooled and named as Batch 1 and 2, respectively, according to CBS after SDS-PAGE (Figure E3A, inset). The 11S globulin Act d 12 from kiwi seeds contained in batch 1 was purified to homogeneity by ion-exchange chromatography (Figure E3B). The purified Act d 12 was visualized as a single protein by CBS after SDS-PAGE under non-reducing conditions and retained its IgE-binding capacity as demonstrated by immunoblotting using sera from kiwifruit allergic patients (Figure E3B, inset). The 2S albumin Act d 13 from kiwi seeds contained in batch 2 was further purified by RP-HPLC. The purified Act d 13 was also visualized as a single monomer of around 12 kDa by CBS after SDS-PAGE under non-reducing conditions that retained the IgE-binding capability as demonstrated by immunoblotting with sera from kiwifruit allergic patients (Figure E3C, inset).

REFERENCES

1. Palomares O, Cuesta-Herranz J, Vereda A, Sirvent S, Villalba M, Rodriguez R. Isolation and identification of an 11S globulin as a new major allergen in mustard seeds. *Ann Allergy Asthma Immunol* 2005; 94:586-92.
2. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193:265-75.
3. Malling HJ. Skin prick testing in biological standardization of allergenic products. *Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M* 1997:157-63.
4. Sirvent S, Palomares O, Vereda A, Villalba M, Cuesta-Herranz J, Rodriguez R. nsLTP and profilin are allergens in mustard seeds: cloning, sequencing and recombinant production of Sin a 3 and Sin a 4. *Clinical and Experimental Allergy* 2009; 39:1929-36.
5. Huecas S, Villalba M, González E, Martínez-Ruiz A, Rodríguez R. Production and detailed characterization of biologically active olive pollen allergen Ole e 1 secreted by the yeast *Pichia pastoris*. *Eur. J. Biochem.* 1999; 261:539-46.
6. Huecas S, Villalba M, Gonzalez E, Martinez-Ruiz A, Rodriguez R. Production and detailed characterization of biologically active olive pollen allergen Ole e 1 secreted by the yeast *Pichia pastoris*. *Eur J Biochem* 1999; 261:539-46.

LEGEND TO FIGURES

FIG E1. IgE-binding profile to home-made total kiwifruit extract of the 55 kiwifruit allergic patients from group 1 (lanes 1 to 28) and group 2 (lanes 29 to 55). C1 and C2 are non-atopic and pollen allergic subjects, respectively.

FIG E2. A. Amino acid sequence alignment of 11S globulin from *Actinidia chinensis* (UNIPROT accession number A0EM47) and the peptides of Act d 12 determined by Edman degradation (ED) and MS/MS. **B.** Amino acid sequence alignment of Sin a 1, 2S albumin from yellow mustard seeds (UNIPROT accession number P15322) and the peptides of Act d 13 determined by Edman degradation (ED) and MS/MS. Identical amino acids are colored in grey.

FIG E3. (A) Elution profile of kiwi seeds extract after a size-exclusion chromatography. Two main proteins of around 51 and 12 kDa were separated into Batch 1 and 2 (shadow in grey). The kiwi seeds extract and the proteins of each batch were visualized by CBS after SDS-PAGE (inset). **(B)** Elution profile of Batch 1 after ion-exchange chromatography. Fractions containing the 11S globulin Act d 12 is shadowed in grey. Purified Act d 12 was visualized by CBS and its IgE-binding capacity was analyzed with sera from kiwifruit allergic patients (inset). **(C)** Elution profile of Batch 2 in RP-HPLC. Fractions containing the 2S albumin Act d 13 is shadowed in grey. Purified Act d 13 was visualized by CBS and its IgE-binding capacity was analyzed with sera from kiwifruit allergic patients (inset).

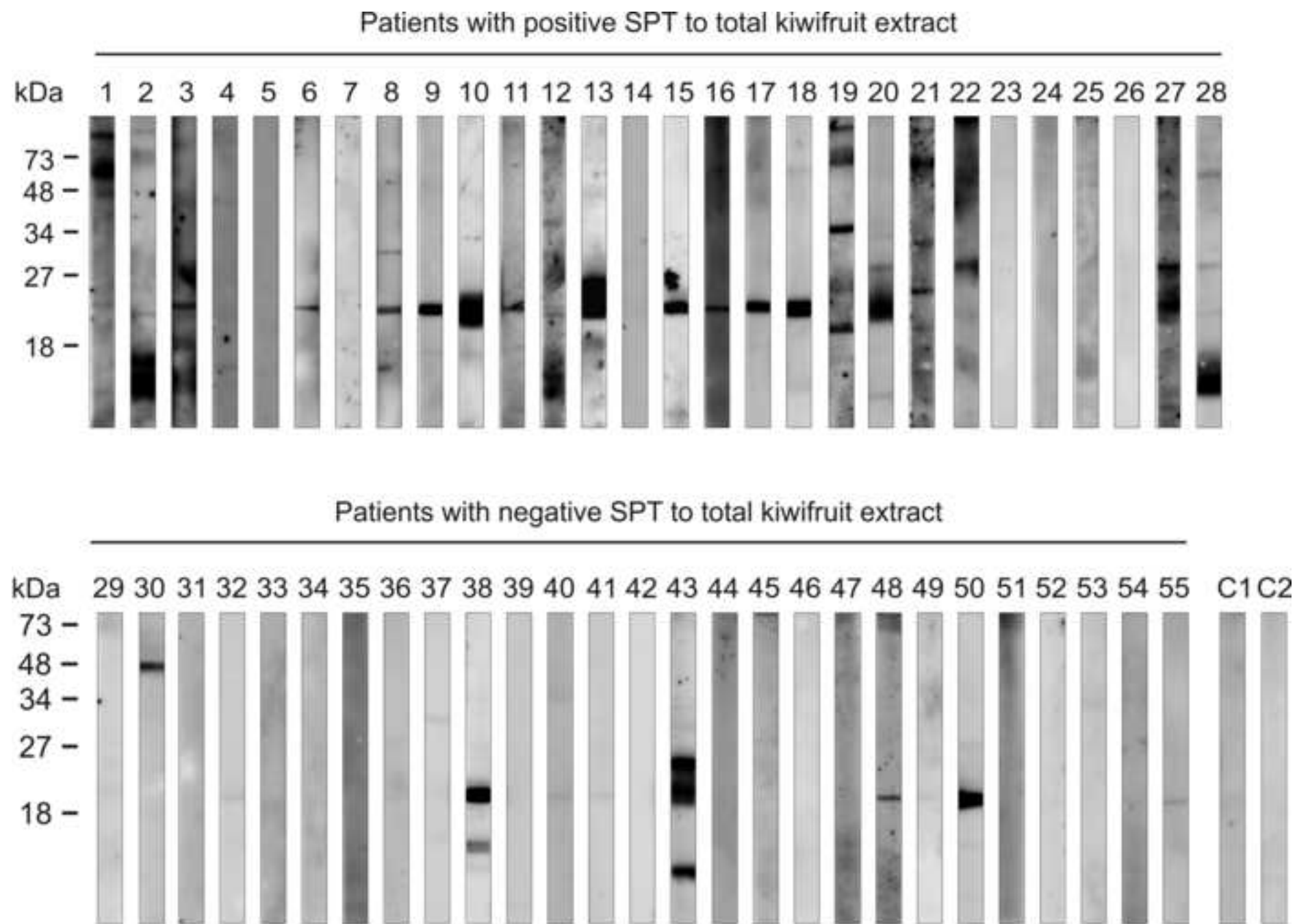
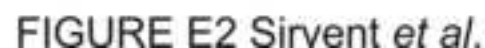


FIGURE E1 Sirvent *et al.*



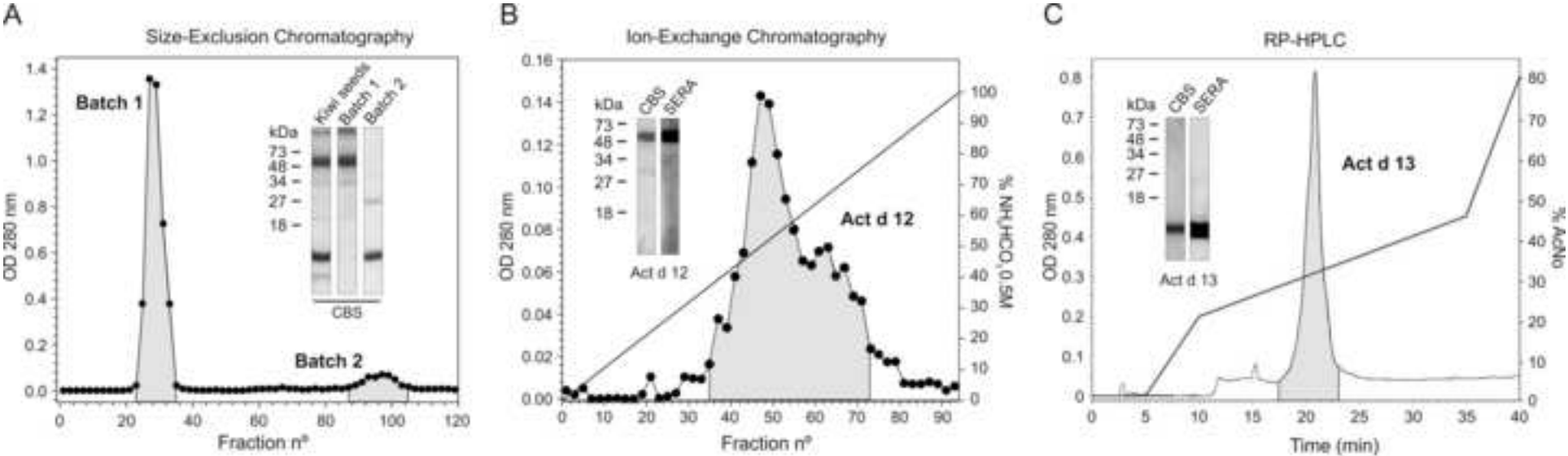


FIGURE E3 Sirvent *et al.*